THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 38, Issue of September 20, pp. 23185–23190, 1996
Printed in U.S.A.

Nonenzymatic Free Radical-catalyzed Generation of Thromboxane-like Compounds (Isothromboxanes) *in Vivo**

(Received for publication, May 30, 1996, and in revised form, July 10, 1996)

Jason D. Morrow, Joseph A. Awad, Aiping Wu, William E. Zackert, Vincent C. Daniel, and L. Jackson Roberts II‡

From the Departments of Medicine and Pharmacology, Vanderbilt University, Nashville, Tennessee 37232-6602

The isoprostanes (IsoPs) are novel bioactive prostaglandin-like compounds produced *in vivo* by free radical-catalyzed peroxidation of arachidonyl-containing lipids. Previously, we have identified IsoPs containing F-type and D- and E-type prostane rings that are formed by reduction and rearrangement of IsoP endoperoxide intermediates, respectively. We now explore whether thromboxane B_2 (TxB_2)-like compounds, termed B_2-isothromboxanes (B_2-IsoTxs), are formed by rearrangement of IsoP endoperoxides. Detection of these compounds was carried out using a stable isotope dilution mass spectrometric assay originally developed for quantification of cyclooxygenase-derived TxB_2. Incubations of arachidonic acid with Fe/ADP/ascorbate for 30 min *in vitro* generated a series of peaks representing putative B_2-IsoTx at levels of 62.4 ± 21.0 ng/mg arachidonate. Using various chemical modification and derivatization approaches, it was determined that these compounds contained hemiacetal ring structures and two double bonds, as would be expected for B_2-IsoTx. Analysis of the compounds by electron ionization mass spectrometry yielded multiple mass spectra similar to those of TxB_2. B_2-IsoTxs are also formed esterified to phospholipids; oxidation of arachidonyl-containing phosphatidylcholine *in vitro* followed by hydrolysis resulted in the release of large amounts of these compounds. To explore whether B_2-IsoTxs are also formed *in vivo*, a well characterized animal model of lipid peroxidation consisting of orogastric administration of CCl_4 to rats was used. Levels of B_2-IsoTx esterified in lipids in the liver increased 41-fold from 2.5 ± 0.5 to 102 ± 30 ng/g of liver. In addition, circulating levels of free compounds increased from undetectable (<5 pg/ml) to 185 ± 30 pg/ml after CCl_4, a 37-fold increase. Thus, we have provided evidence that IsoTxs constitute another novel class of eicosanoids produced *in vivo* nonenzymatically by free radical-catalyzed lipid peroxidation. These studies thus expand our understanding of products of lipid peroxidation formed *in vivo* from the free radical-catalyzed peroxidation of arachidonic acid.

Free radical-catalyzed lipid peroxidation has been implicated in the pathogenesis of a wide variety of human disorders (1–4). Nonetheless, much remains to be understood about the mechanisms of oxidant injury *in vivo*. Previously, we reported the discovery that a series of prostaglandin (PG)[1] F_2-like compounds (F_2-isoprostanes (F_2-IsoPs)) capable of exerting potent biological activity are produced *in vivo* in humans as products of the free radical-catalyzed peroxidation of arachidonic acid (5). Formation of these compounds occurs independently of the cyclooxygenase enzyme, which had heretofore been considered obligatory for endogenous prostanoid biosynthesis. Circulating levels of these compounds increase dramatically in animal models of free radical injury, and quantification of F_2-IsoPs has proven to be an important advance in our ability to assess oxidant stress *in vivo* (5, 6). Formation of F_2-IsoPs proceeds through intermediates comprising four positional peroxyl radical isomers, which undergo endocyclization to yield PGG_2-like bicycloendoperoxides. These are then reduced to F-ring IsoPs. F_2-IsoPs are initially formed *in situ* from arachidonic acid esterified in phospholipids and are subsequently released preformed by a phospholipase (6, 7). This mechanism of formation is in contradistinction to the formation of cyclooxygenase-derived prostanoids in which arachidonic acid esterified in phospholipids must be released prior to oxygenation.

More recently, we reported that IsoPs that are PGD_2- and PGE_2-like compounds (D_2/E_2-IsoPs) also are produced *in vivo* from rearrangement of isoprostane endoperoxides (8). Like F-ring compounds, they are formed *in situ* on phospholipids, their formation increases markedly in animal models of oxidant injury, and they exert potent bioactivity. Because Tx can also be formed by nonenzymatic rearrangement of cyclooxygenase-derived PGH_2 (9), we explored whether Tx-like compounds can also be generated as rearrangement products of the IsoP endoperoxide intermediates. We present evidence that TxB_2-like compounds are, in fact, produced both *in vitro* and *in vivo* and that they are present both esterified to phospholipids and in the free form. Because these compounds are isomeric to cyclooxygenase-derived TxB_2, they henceforth will be referred to as B_2-IsoTx.

EXPERIMENTAL PROCEDURES

Reagents—Methoxyamine HCl, $FeCl_3$, ascorbate, ADP, pentafluorobenzyl (PFB) bromide, diisopropylethylamine, and *Apis mellifera* venom phospholipase A_2 were obtained from Sigma. Dimethylformamide, undecane, and sodium borohydride were obtained from Aldrich. *N,O*-Bis(trimethylsilyl)trifluoroacetamide and *N*-trimethylsilylimidazole were obtained from Supelco (Bellefonte, PA). [$^2H_9$]*N,O*-Bis(trimethylsilyl)trifluoroacetamide and [$^2H_3$]methoxyamine HCl were purchased from Regis Chemical Co. (Morton Grove, IL). All organic solvents were obtained from Baxter Healthcare (Burdick and Jackson Brand, McGraw Park, IL). C-18 and silica Sep-Paks were purchased from Waters Associates (Milford, MA). TLC was performed on Silica gel

* This work was supported by National Institutes of Health Grants GM42056, GM15431, and DK48831. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Medicine and Pharmacology, Vanderbilt University, Nashville, TN 37232-6602. Tel.: 615-343-1816; Fax: 615-322-4707; E-mail: jack.roberts@mcmail.vanderbilt.edu.

[1] The abbreviations used are: PG, prostaglandin; IsoP, isoprostane; IsoTx, isothromboxane; F_2-IsoP, PGF_2-like IsoP; D_2/E_2-IsoP, PGD_2- and PGE_2-like IsoP; Tx, thromboxane; GC, gas chromatography; NICI, negative ion chemical ionization; MS, mass spectrometry; PFB, pentafluorobenzyl; TMS, trimethylsilyl; EI, electron ionization.

60ALK6D plates (Whatman). [$^2H_3$]Thromboxane B_2 and other deuterated prostaglandin standards were obtained from Cayman Chemical (Ann Arbor, MI). Arachidonic acid and 1-palmitoyl,2-arachidonyl phosphatidylcholine were purchased from Avanti Polar Lipids (Alabaster, AL).

Analysis of Isothromboxanes—IsoTxs were analyzed by gas chromatography (GC)/negative ion chemical ionization (NICI) mass spectrometry (MS) using a modification of methods described previously for the analysis of TxB_2 (10). Briefly, 1.5 ng of [$^2H_3$]TxB_2 internal standard was initially added to a biological fluid and adjusted to pH 3 with 1 M HCl. The sample was applied to a C-18 Sep-Pak cartridge that had been prewashed with 5 ml of methanol and 5 ml of H_20 (pH 3). The cartridge was then washed with 10 ml of H_2O (pH 3) followed by 10 ml heptane, and compounds were eluted with 10 ml of ethyl acetate and evaporated to dryness under nitrogen. Compounds were subsequently methoximated by treatment with 250 μl of a 2% solution of aqueous methoxyamine HCl for 30 min at room temperature. Compounds were extracted with 1 ml of ethyl acetate, and the organic layer was evaporated under nitrogen. Compounds were then converted to a PFB ester by addition of 40 μl of a 10% solution of PFB bromide in acetonitrile and 20 μl of 10% diisopropylethylamine in acetonitrile and incubated for 30 min at 37 °C. Reagents were dried under nitrogen, and the residue was reconstituted in 30 μl of chloroform and 20 μl of methanol and chromatographed on a silica TLC plate to the top in a solvent system of ethyl acetate: methanol (98:2, v/v). The *O*-methyloxime and PFB ester derivative of TxB_2 (approximately 5 μg), was chromatographed on a separate lane and visualized with 10% phosphomolybdic acid in ethanol by heating. The R_F of the derivatized TxB_2 standard in this solvent system was ~0.46. Compounds migrating in the region 1.5 cm above and below the standard were scraped from the TLC plate, extracted with 1 ml of ethyl acetate, and dried under nitrogen.

Following TLC purification, compounds were converted to trimethylsilyl (TMS) ether derivatives by addition of 20 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide and 10 μl of dimethylformamide. The sample was incubated at 37 °C for 10 min and then dried under nitrogen. The residue was redissolved for GC/MS analysis in 10 μl of undecane, which had been stored over a bed of calcium hydride. GC/NICI MS was carried out on a Nermag R10-10C mass spectrometer interfaced with a Digital DEC-PDP computer. GC was performed using a 15-m, 0.25-μm film thickness, DB-1701 fused silica capillary column (J & W Scientific, Folsom CA). The column temperature was programmed from 190° to 300 °C at 20 °C/min. The major ion generated in the NICI mass spectrum of the PFB ester, *O*-methyloxime, and TMS ether derivative of TxB_2, which would be the same ion generated by IsoTx, was the *m/z* 614 carboxylate anion M − 181 (M − $^{\cdot}CH_2C_6F_5$). The corresponding ion generated by the [$^2H_3$]TxB_2 internal standard was *m/z* 617. Levels of endogenous B_2-IsoTx in a biological sample were calculated from the ratio of the area under the *m/z* 614 chromatographic peaks to the *m/z* 617 chromatographic peak. In some experiments, compounds were reacted with trimethylsilyimidazole, subjected to catalytic hydrogenation, or reduced with sodium borohydride following TLC purification as described (7). IsoTxs were also analyzed by GC/electron ionization (EI) MS as methyl ester *O*-methyloxime and TMS ether derivatives. Purification and derivatization of compounds for analysis by GC/EI MS were as noted above, except the methyl ester derivatives were formed by treatment of compounds with excess ethereal diazomethane (7).

Analysis of F_2- and D_2/E_2-IsoPs—Purification, derivatization, and analysis of F_2-IsoPs and D_2/E_2-IsoPs by GC/NICI MS were performed as described (7, 8) Quantification of either F_2-IsoPs or D_2/E_2-IsoPs in the present studies differed from previous reports in that the amounts of endogenous IsoPs were determined by comparing the ratios of the area under the chromatographic peaks representing endogenous material to that of the respective standard.

Extraction, Purification, and Hydrolysis of Phospholipids—1-Palmitoyl-2-arachidonylphosphatidylcholine oxidized *in vitro* or lipids from livers of CCl_4-treated rats were extracted as described (6, 11) Depending on the experiment, 0.005% butylated hydroxytoluene was added to the lipid extracts during the extraction procedure. The lipid extracts (containing approximately 1 μmol of phospholipid) were then hydrolyzed by chemical saponification or by reaction with *A. mellifera* venom phospholipase A_2 (approximately 200 μg) as described (6, 8) and subsequently analyzed for free B_2-IsoTx. As a positive control for phospholipase A_2 activity, phosphatidylcholine containing [^{3}H]arachidonate in the *sn*-2 position was added to the incubation mixture, and the percent of radiolabeled arachidonate released was determined as described (6, 8). In all experiments, >95% of esterified [^{3}H]arachidonate was released.

Oxidation of Arachidonic Acid and Arachidonyl Phosphatidylcholine in Vitro—Arachidonic acid and arachidonyl phosphatidylcholine were oxidized for 30 min using a Fe/ADP/ascorbate oxidizing system as described (12).

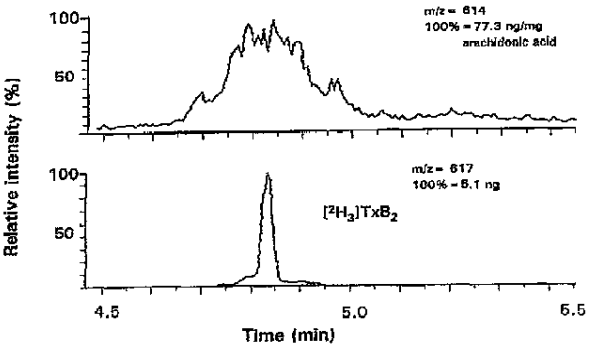

FIG. 1. **Analysis of oxidized arachidonic acid for B_2-IsoTx by GC/NICI MS.** The peak in the *m/z* 617 ion current chromatogram represents the [$^2H_3$]TxB_2 internal standard. In the *m/z* 614 ion current chromatogram are a series of peaks consistent with the presence of B_2-IsoTx compounds. Levels of compounds are based on a comparison of the ratio of the area under the series of peaks in the *m/z* 614 chromatogram with the area under the peak in the *m/z* 617 chromatogram.

Animal Model of Free Radical-induced Lipid Peroxidation—Free radical-catalyzed lipid peroxidation was induced in rats by intragastric administration of CCl_4 as described previously (13). At various time intervals, animals were sacrificed, and the livers were removed, snap frozen in liquid N_2, and either processed immediately or stored at −70 °C. In some experiments, animals were pretreated with 5 mg/kg indomethacin at 24, 12, and 2 h prior to receiving CCl_4. This has been previously shown to inhibit the cyclooxygenase >90% (14).

RESULTS

Evidence of the Formation of Isothromboxanes in Vitro—Previously, we had shown that oxidation of arachidonic acid *in vitro* results in the formation of large amounts of both F_2-IsoPs and D_2/E_2-IsoPs (7, 8). Thus, we initially explored whether IsoTxs are also formed *in vitro* by analyzing arachidonic acid that had been oxidized with Fe/ADP/ascorbic acid. As described above, IsoTxs were detected using an assay originally developed for cyclooxygenase-derived TxB_2. The selected ion current chromatograms obtained from this analysis monitoring *m/z* 614 for B_2-IsoTx and *m/z* 617 for the [$^2H_3$]TxB_2 internal standard are shown in Fig. 1. When analyzed in an analogous fashion, F_2- or D_2/E_2-IsoPs are detected as a series of peaks eluting from the GC over approximately a 30-s interval. Similarly, in the upper *m/z* 614 chromatogram in Fig. 1, a series of peaks is present, which eluted from the GC column over an approximate 20–30-s interval. The peak in the lower *m/z* 617 chromatogram represents the coeluting *syn*- and *anti*-*O*-methyloxime isomers of the [$^2H_3$]TxB_2 standard. Quantification of compounds in the *m/z* 614 chromatogram is based on a comparison of the ratios of the area under the sum of peaks in the *m/z* 614 chromatogram to the area under the peak representing the internal standard in the *m/z* 617 chromatogram. The amount of the compounds in the *m/z* 614 chromatogram was calculated to be 77.3 ng/mg arachidonic acid. Four other incubations of arachidonic acid were analyzed in a similar fashion, and for all five incubations, the mean value of presumed B_2-IsoTx was 62.4 ± 21.0 ng/mg arachidonic acid. For comparison, in the same incubations, the yield of F_2-IsoPs was 232 ± 64 ng/mg arachidonic acid, and the yield of D_2/E_2-IsoPs was 1269 ± 446 ng/mg arachidonic acid (n = 5). Nearly identical amounts of presumed B_2-IsoTx (84 ± 31 ng/mg arachidonic acid, n = 5) were obtained when arachidonic acid esterified to phospatidylcholine was used as a substrate and B_2-IsoTx was quantified after base hydrolysis or hydrolysis with bee venom phospholipase A_2.

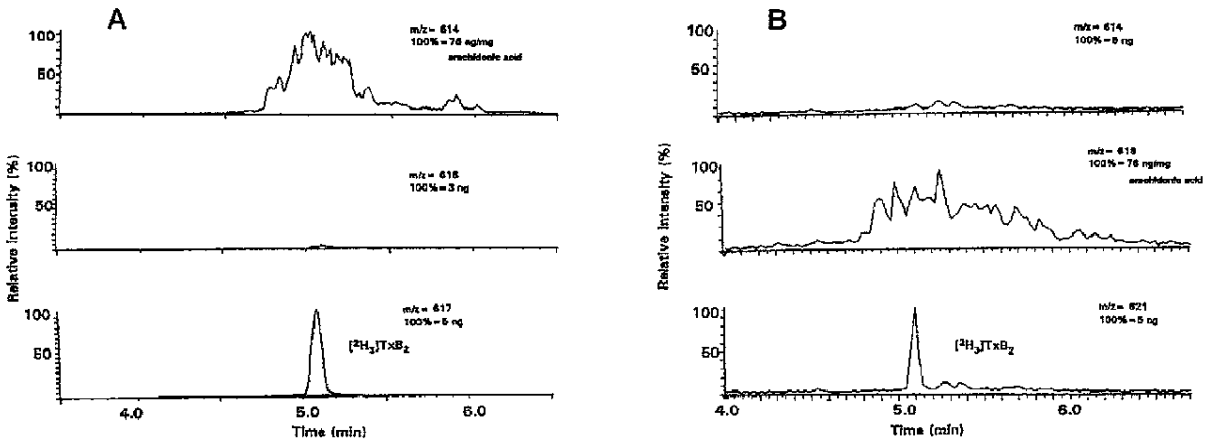

FIG. 2. **Catalytic hydrogenation of putative B_2-IsoTx obtained from arachidonic acid oxidized *in vitro*.** *A*, GC/NICI MS analysis of compounds not subjected to catalytic hydrogenation. Peaks in the *m/z* 614 chromatogram represent putative B_2-IsoTx, and the peak in the *m/z* 617 chromatogram represents the $[^2H_3]TxB_2$ internal standard. In addition, no compounds are present in the *m/z* 618 chromatogram. *B*, analysis of compounds following hydrogenation. Both the internal standard and *m/z* 614 peaks in *A* have shifted up 4 Da following hydrogenation, indicating the presence of two double bonds. The pattern of *m/z* 618 peaks in *B* differs somewhat from the pattern of *m/z* 614 peaks in *A*. This is likely attributed to different GC characteristics of the compounds in which double bonds have been reduced.

The finding that large quantities of a series of compounds were formed during oxidation of arachidonic acid *in vitro* that had TLC and GC/MS properties similar to those of TxB_2 would be consistent with their being B_2-IsoTx. However, additional experimental approaches were used to obtain further evidence that the compounds detected in oxidized arachidonic acid were B_2-IsoTxs. First, no peaks were present when *m/z* 613 was monitored, indicating that the *m/z* 614 peaks were not natural isotope peaks of compounds generating an ion of less than 614 Da. When the compounds were analyzed as $[^2H_9]$TMS ether derivatives, the *m/z* 614 peaks all shifted upward 27 Da, indicating that the compounds have three hydroxyl groups. When the compounds were analyzed as $[^2H_3]$*O*-methyloxime derivatives, the *m/z* 614 peaks all shifted upward 3 Da, indicating that they contain one carbonyl group. When the compounds were analyzed following catalytic hydrogenation, there was a disappearance of the *m/z* 614 peaks and the appearance of new intense peaks 4 Da higher at *m/z* 618 (Fig. 2). No peaks were detected at *m/z* 616 or 620. This indicated that all of the compounds contained two double bonds. Collectively, these results indicated that the compounds represented by the *m/z* 614 peaks contain the same functional groups and the number of double bonds expected for the PFB ester, *O*-methyloxime, and TMS ester derivative of B_2-IsoTx.

A unique feature of TxB_2 is that it contains a hemiacetal ring, which exists in aqueous solution in an equilibrium between open and closed forms. Thus, powerful evidence that the compounds detected were B_2-IsoTxs would be to demonstrate that these compounds contain a hemiacetal ring. Such evidence can be obtained using different derivatization and chemical modification approaches (15). As shown in Fig. 3*A*, if putative B_2-IsoTxs are first reacted with methoxyamine, derivatives will be formed in which the hemiacetal ring is open. Subsequent conversion to PFB ester and TMS ether derivatives would be expected to result in a series of compounds with a major fragment ion of 614 Da (M − 181 and M − $^{\cdot}CH_2C_6F_5$) when analyzed by GC/NICI MS. The selected ion monitoring analysis of presumed IsoTx derivatized in this manner has been previously discussed and is shown in Fig. 1. If, on the other hand, as shown in Fig. 3*B*, the treatment with methoxyamine is omitted, and the compounds are converted to PFB ester and TMS ether derivatives, the hemiacetal ring will remain closed. Derivatives of these compounds would be expected

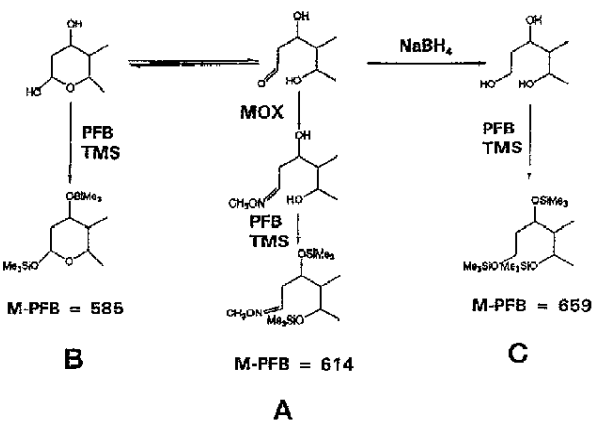

FIG. 3. **Three different derivatization and reduction approaches used to provide evidence of the presence of a hemiacetal ring in the putative B_2-IsoTx compounds formed from the oxidation of arachidonic acid *in vitro*.** The major fragment ion expected for each derivative when analyzed by NICI MS is shown at the *bottom* of each pathway.

to generate major fragment ions of 585 Da (M − 181) when analyzed by NICI MS. Results using this derivatization approach are shown in Fig. 4*A*. As is evident, in the upper *m/z* 585 chromatogram, a series of chromatographic peaks are present that elute at a similar retention time to the $[^2H_3]TxB_2$ internal standard represented in the lower *m/z* 588 chromatogram. Finally, as shown in Fig. 4*C*, if the carbonyl at C-11 in the open ring form is first reduced with $NaBH_4$ followed by conversion to PFB ester and TMS ether derivatives, the major M − 181 fragment ion would be generated at 659 Da. Results of this analysis are shown in Fig. 4*B*. Again, a series of *m/z* 659 peaks elute from the GC at a retention time similar to this derivative of the TxB_2 internal standard. Collectively, the results of these studies provide additional significant evidence that these compounds contain a hemiacetal ring as does TxB_2.

Analysis of B_2-IsoTx by EI MS—To obtain more direct evidence that the compounds detected by NICI MS were B_2-IsoTx, the compounds were analyzed as methyl ester, *O*-methyloxime, and TMS ether derivatives by EI MS. The results of this analysis yielded a series of compounds eluting over approximately a

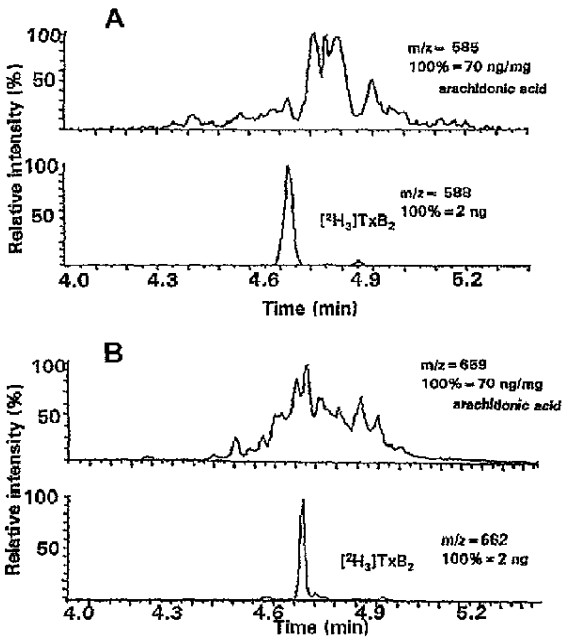

FIG. 4. **GC/NICI MS analysis of putative B_2-IsoTx from arachidonic acid oxidized *in vitro*.** *A*, analysis of compounds as PFB ester and TMS ether derivatives. In the *lower m/z* 588 chromatogram is the derivatized $[^2H_3]TxB_2$ internal standard. In the *upper m/z* 585 chromatogram are a series of peaks eluting over a 30-s interval. *B*, analysis of compounds after reduction with $NaBH_4$ followed by conversion to PFB ester and TMS ether derivatives. The peak representing this derivative of the $[^2H_3]TxB_2$ internal standard is shown in the *lower m/z* 662 chromatogram. The series of peaks in the *upper m/z* 659 chromatogram represents presumed B_2-IsoTx compounds. The pattern of peaks in Fig. 1, *A* and *B*, likely differs due to different GC characteristics of the various derivatives.

20-s period from the capillary GC column, which yielded mass spectra with characteristics of the EI mass spectrum of TxB_2. One of the mass spectra obtained from a major peak is shown in Fig. 5. Other mass spectra obtained from the analysis of the other peaks were similar to that shown in Fig. 5, except that either the relative abundance of some of the fragment ions varied or some of the lower molecular weight fragment ions were different. In the mass spectrum shown, there is a prominent ion at *m/z* 629, representing the molecular ion. Other characteristic ions present are *m/z* 614 (M − 15, loss of $^{\cdot}CH_3$), *m/z* 598 (M − 31, loss of $^{\cdot}OCH_3$), *m/z* 539 (M − 90, loss of $(Me_3)_3SiOH$), *m/z* 524 (loss of 90 + 15), *m/z* 508 (loss of 90 + 31), *m/z* 418 (loss of 2 × 90 + 31), *m/z* 398 (loss of $^{\cdot}CH_2CH{=}CH(CH_2)_3COOCH_3$ + 90), *m/z* 392 (M − 57 − (2 × 90), loss of $^{\cdot}CH_2(CH_2)_2CH_3$ + (2 × 90)), *m/z* 369 [M − 173 − 87, loss of $^{\cdot}CH_2(OSi(Me_3)_3(CH_2)_4CH_3)$ + $^{\cdot}CH_2CH_2COOCH_3$), *m/z* 243, $((Me_3)_3SiO^+{=}CHCH_2CH{=}CH(CH_8)_3COOCH_3)$ and *m/z* 174 $((Me_3)_3SiO^+{=}CHCH_2CH{=}NOCH_3)$. Of particular interest is the major fragment ion of 243 Da. This ion is not present in the mass spectrum of this derivative of cyclooxygenase-derived TxB_2. However, this is an expected ion resulting from α cleavage of the trimethoxysiloxy substitutent at C-8, as depicted in the regioisomer shown in Fig. 5 (7, 16). Thus, this EI mass spectral data provide additional confirmatory evidence for the formation of IsoTxs by nonenzymatic peroxidation of arachidonic acid.

Analysis for the Presence of B_2-IsoTx Esterified to Phospholipids in Vivo—Since the above results suggested that IsoTxs could be formed *in vitro*, we investigated whether these compounds may also be formed *in vivo*. Previously, we had shown

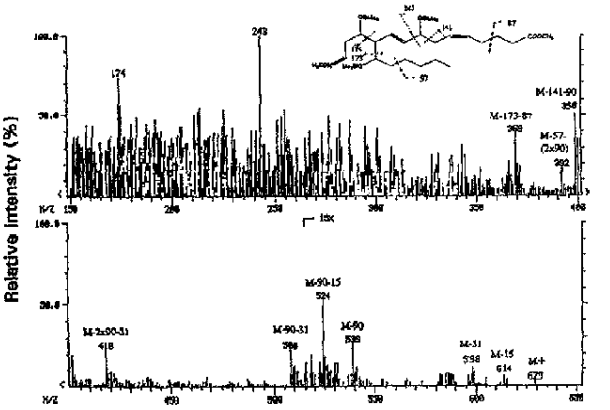

FIG. 5. **A representative electron ionization mass spectrum of a B_2-IsoTx compound derived from oxidation of arachidonic acid *in vitro* with Fe/ADP/ascorbate.** Compounds were analyzed as a methyl ester, *O*-methyloxime, and TMS ether derivative.

that F_2-IsoPs and D_2/E_2-IsoPs are initially formed *in situ* from arachidonic acid esterified in tissue phospholipids and subsequently released preformed (6, 8). Therefore, we examined whether IsoTxs are also formed esterified in phospholipids in livers of rats that had been treated with CCl_4 to induce lipid peroxidation. To investigate this, lipids were extracted from the livers, subjected to hydrolysis using methanolic potassium hydroxide, and analyzed as free compounds. The results of this analysis are shown in Fig. 6. A series of *m/z* 614 peaks was present in a pattern very similar to that obtained from analysis of arachidonic acid oxidized *in vitro*, although the relative abundances of the various peaks differ slightly (*cf.* Fig. 1). Essentially identical results were obtained when phospholipids were hydrolyzed enzymatically with phospholipase A_2 from *A. mellifera* (data not shown).

Table I compares the amounts of the B_2-IsoTxs with D_2/E_2-IsoPs and F_2-IsoPs measured following hydrolysis of lipids from the same livers of both untreated and CCl_4-treated rats. The quantities of B_2-IsoTx measured following hydrolysis of lipids from livers of CCl_4-treated rats were 41-fold higher than those in untreated rats. Levels of free B_2-IsoTx measured in lipid extracts that were not subjected to hydrolysis were <1% of the levels measured following hydrolysis (n = 4), suggesting that the compounds detected following saponification were released from an acyl linkage on phospholipids. Pretreatment of animals with indomethacin prior to CCl_4 administration with a dosage regimen previously shown to inhibit cyclooxygenase activity by >90% (14) did not affect levels of the compounds measured ($p > 0.7$, Student's t test; n = 4), indicating that the cyclooxygenase enzyme is not involved in their formation. Previously we had shown that butylated hydroxytoluene markedly suppresses the formation of F_2-IsoPs by autoxidation *in vitro* (7). The presence of butylated hydroxytoluene (0.005%) in the extraction solution, however, did not affect levels of IsoTx measured ($p > 0.6$; n = 4), arguing that these compounds are not formed *ex vivo* by autoxidation during sample processing.

Experiments were then carried out to obtain further evidence of the identity of the compounds represented by the *m/z* 614 peaks in Fig. 6 as B_2-IsoTx. First, analysis of compounds obtained from the hydrolysis of liver extracts as a $[^2H_9]TMS$ ether derivative resulted in a shift of each of the *m/z* 614 peaks upwards of 27 Da, indicating the presence of three hydroxyl groups. Analysis as $[^2H_3]O$-methyloxime derivatives resulted in a shift of the *m/z* 614 peaks upwards of 3 Da, indicating the presence of one carbonyl group. When the compounds were analyzed following catalytic hydrogenation, there was a disap-

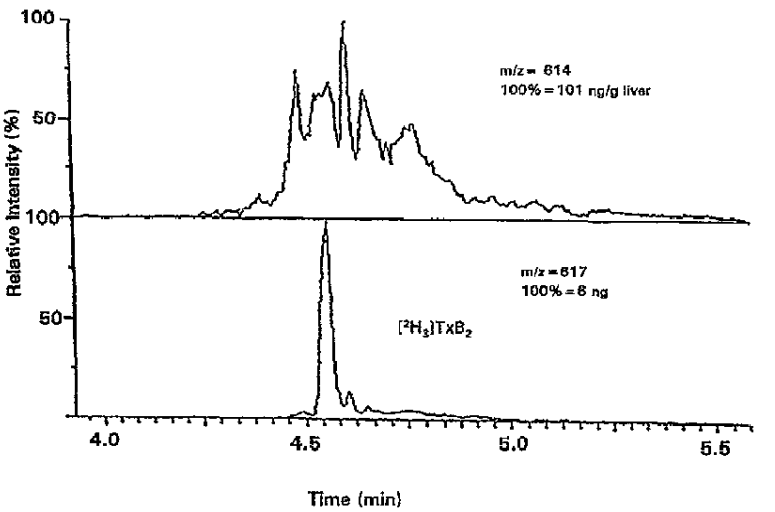

FIG. 6. **Analysis for B_2-IsoTx by GC/NICI MS following hydrolysis of a lipid extract from the liver of a rat treated with CCl_4 to induce lipid peroxidation.** A series of peaks are present in the *m/z* 614 ion current chromatogram representing B_2-IsoTx; the *m/z* 617 chromatogram represents the $[^2H_3]TxB_2$ internal standard.

TABLE I
Comparison of levels of B_2-IsoTx and IsoP in hydrolyzed lipid extracts from liver tissue of rats with and without CCl_4 treatment (n = 5 animals/group)

Rats	B_2-IsoTxs	D_2/E_2-IsoPs	F_2-IsoPs
		ng/g liver	
Untreated	2.5 ± 0.5	1.9 ± 0.3	4.6 ± 0.7
CCl_4 treated	102 ± 30	161 ± 37	672 ± 179

pearance of the *m/z* 614 peaks and the appearance of new intense peaks 4 Da higher at *m/z* 618. No peaks were detected at *m/z* 616 or 620. This indicated that all of the compounds contained two double bonds. Furthermore, analysis of the compounds using the different derivatization approaches and chemical modification as outlined in Fig. 3 indicated the presence of a hemiacetal ring, as was found in the compounds generated *in vitro* (Fig. 4). Collectively, these results indicated that the compounds generated *in vivo* represented by the *m/z* 614 peaks contain a hemiacetal ring and the same functional groups and number of double bonds as would be expected for B_2-IsoTx.

Analysis for the Presence of Free B_2-IsoTx in the Circulation in CCl_4-treated Rats—We have previously demonstrated that F_2- and D_2/E_2-IsoPs are initially formed esterified to tissue phospholipids in CCl_4-treated rats and subsequently released into the circulation preformed (6, 8, 17). Thus, we examined whether increased concentrations of these IsoTxs could also be detected free in the circulation of rats 4 h following administration of CCl_4 to induce lipid peroxidation. In these studies, animals were pretreated with indomethacin to inhibit cyclooxygenase-derived TxB_2 from blood elements. IsoTxs could not be detected in plasma from normal rats that had not been treated with CCl_4 (lower limit of detection, 5 pg/ml; n = 4). However, following treatment of rats with CCl_4, B_2-IsoTx were detected in plasma at concentrations of 185 ± 30 pg/ml (n = 4), representing an increase of 37-fold. The pattern of peaks was essentially identical to that shown in Fig. 6. We have previously shown that increases in levels of F_2-IsoPs esterified to circulating lipids parallel increases in free levels after administration of CCl_4 (17). Therefore, we also quantified levels of B_2-IsoTxs esterified in circulating plasma lipids and found that CCl_4 administration increased levels from 13 ± 12 to 237 ± 63 pg/ml (n = 4). In summary, these studies support the concept that B_2-IsoTxs are formed *in situ* on phospholipids and subsequently released preformed into the circulation.

DISCUSSION

These studies report the discovery that thromboxane-like compounds, termed IsoTxs, are formed both *in vitro* and *in vivo* by nonenzymatic free radical-catalyzed peroxidation of arachidonic acid. Analogous to the formation of F_2-IsoPs and D_2/E_2-IsoPs, B_2-IsoTxs are also formed *in situ* esterified to phospholipids and released in the free form, presumably by a phospholipase. A pathway for the formation of these compounds is outlined in Fig. 7. It is identical to that outlined previously for the formation of F_2-IsoPs and D_2/E_2-IsoPs involving the formation of the bicyclic endoperoxide intermediates (6, 8). In the formation of IsoTx, however, the endoperoxides undergo rearrangement to form TxA_2-like compounds, termed A_2-IsoTxs, which then rapidly decompose to more stable TxB_2-like molecules, termed B_2-IsoTxs. Analogous to the formation of F_2-IsoPs, four regioisomers of B_2-IsoTxs are formed, each of which can theoretically comprise eight racemic pairs of diastereomers.

Although B_2-IsoTx can be generated both *in vitro* and *in vivo* from arachidonic acid, the chemistry involved in the conversion of isoprostane endoperoxides to IsoTx is not entirely clear. It may, however, be similar to that proposed for the conversion of the cyclooxygenase-derived endoperoxide PGH_2 to TxA_2 by the enzyme thromboxane synthase (9). Thromboxane synthase is a cytochrome P450 enzyme containing a catalytic iron moeity at its active site. Hecker and Ullrich (9) have proposed that the formation of TxA_2 initially involves complexing of the Fe^{3+} in the enzyme active site with the oxygen at C-9 on the endoperoxide PGH_2. This is followed by homolytic scission of the endoperoxide bond, leading to formation of an alkoxyl radical. Subsequently, β scission of the C-11—C-12 bond occurs, followed by rearrangement of the molecule to form TxA_2, which then rapidly decomposes to TxB_2. A similar mechanism might also explain the nonenzymatic formation of B_2-IsoTx from the iron-catalyzed peroxidation of arachidonic acid *in vitro*. Arguing against this mechanism, however, is the observation by Hecker and Ullrich (9) that reaction of inorganic Fe^{+3} with PGH_2 does not result in the formation of significant quantities of TxB_2. In those studies, however, Hecker and Ullrich (9) did find that large amounts of TxB_2 were generated from PGH_2 if iron was present complexed in a porphyrin such as hemin. Thus, it is possible that the formation of IsoTx *in vivo* might be catalyzed by porphyrin-containing compounds or Fe-containing enzymes, including Tx synthase. On the other hand, the fact that large amounts of B_2-IsoTx can be formed *in vitro* when

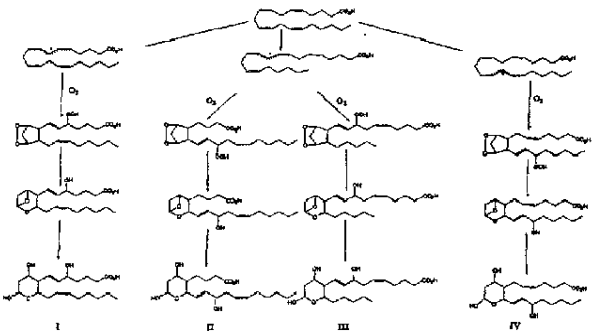

FIG. 7. **Outline of the mechanism involved in the formation of B_2-IsoTxs.** Four regioisomers are produced, each of which can theoretically comprise eight racemic diastereomers. For simplicity, stereochemical orientation is not indicated.

arachidonic acid is oxidized with Fe/ADP/ascorbate would suggest that complexed iron or Fe-containing enzymes are not necessary for the formation of IsoTx.

It should be noted that the quantities of B_2-IsoTxs that are formed *in vivo* are only slightly less than the amounts of D_2/E_2-IsoPs generated. Since the levels of many of the individual IsoPs in normal human biological fluids are at least an order of magnitude higher than cyclooxygenase-derived prostaglandins, the amounts of IsoTx that are produced *in vivo* are not trivial. We have previously reported that both F- and D/E-ring IsoPs possess potent biological activities (5, 8, 18–20). Whether IsoTx possess biological activity will be difficult to ascertain. It is reasonable to assume that if IsoTx possess bioactivity, such activity would reside with the TxA-ring compounds rather than the TxB-ring compounds, analagous to cyclooxygenase-derived Txs, of which TxA_2 is bioactive but TxB_2 is devoid of biological activity. However, because the TxA-ring is extremely unstable, undergoing rapid hydrolysis to form the TxB-ring, isolation of A_2-IsoTx for biological testing would be difficult, if not impossible.

It should also be mentioned that there are potentially important biological ramifications associated with the formation of IsoTx esterified in phospholipids. We previously reported that molecular modeling of phospholipids with F_2-IsoPs esterified at the *sn*-2 position revealed them to be extremely distorted molecules (6). Thus, the formation of isoprostane-containing phospholipids in settings of oxidant stress may have deleterious effects on membrane fluidity and integrity, well recognized sequelae of oxidant injury (21). Since we have now discovered that, in addition to F_2-IP and D_2/E_2-IsoPs, IsoTxs are also formed esterified to phospholipids in large quantities, the total quantities of phospholipids containing products of the isoprostane pathway that may be formed in settings of free radical injury are substantially greater than previously thought.

In summary, we report the discovery that IsoTxs are formed *in vivo* as products of nonenzymatic free radical-catalyzed lipid peroxidation. Analogous to the formation of F_2-IsoPs and D_2/E_2-IsoPs, IsoTxs are formed *in situ* esterified to phospholipids and subsequently released in free form. Further understanding the biological consequences of the formation of these novel compounds and mechanisms by which they are formed may provide valuable insights into the pathophysiology of oxidant injury.

REFERENCES

1. Halliwell, B., and Gutteridge, J. M. C. (1990) *Methods Enzymol.* **186,** 1–85
2. Southorn, P. A., and Powis, G. (1988) *Mayo Clin. Proc.* **63,** 390–408
3. Ames, B. N. (1983) *Science* **221,** 1256–1264
4. Harman, D. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 7124–7128
5. Morrow, J. D., Hill, K. E., Burk, R. F., Nammour, T. M., Badr, K. F., and Roberts, L. J. I. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9383–9387
6. Morrow, J. D., Awad, J. A., Boss, H. J., Blair, I. A., and Roberts, L. J., II. (1992) *Proc. Natl. Acad. Sci U. S. A.* **89,** 10721–10725
7. Morrow, J. D., Harris, T. M., and Roberts, L. J. I. (1990) *Anal. Biochem.* **184,** 1–10
8. Morrow, J. D., Minton, T. A., Mukundan, C. R., Campbell, M. D., Zackert, W. E., Daniel, V. C., Badr, K. F., Blair, I. A., and Roberts, L. J., II. (1993) *J. Biol. Chem.* **269,** 4317–4326
9. Hecker M., and Ullrich V. (1989) *J. Biol. Chem.* **264,** 141–150
10. Parsons, W. G., and Roberts L. J. (1988) *J. Immunol.* **141,** 2413–2419
11. Radin N. S. (1969) *Methods Enzymol.* **14,** 245–248
12. Longmire A. W., Swift, L. L., Roberts, L. J., Awad, J. A., Burk, R. F., and Morrow, J. D. (1994) *Biochem. Pharmacol.* **47,** 1173–1177
13. Burk R. F., and Lane, J. M. (1979) *Toxicol. Appl. Pharmacol.* **50,** 467–478
14. Jackson E. K. (1989) *J. Pharmacol. Exp. Ther.* **250,** 9–21
15. Roberts, L. J., II, Sweetman, B. J., and Oates, J. A. (1981) *J. Biol. Chem.* **256,** 8384–8393
16. Waugh R. J., and Murphy R. C. (1996) *J. Am. Soc. Mass Spectrom.* **1,** 490–499
17. Morrow, J. D., Awad, J. A., Kato, T., Takahashi, K., Badr, K. F., Roberts, L. J., II., and Burk, R. F. (1992) *J. Clin. Invest.* **90,** 2502–2507
18. Takahashi, K., Nammour, T. K., Fukunaga, M., Ebert, J., Morrow, J. D., Roberts, L. J., II., Hoover, R. L., and Badr, K. F. (1992) *J. Clin. Invest.* **90,** 136–141
19. Banerjee, M., Kang, K. H., Morrow, J. D., Roberts, L. J., and Newman, J. H. *Am. J. Physiol.* **263,** H660–H663
20. Fukunaga, M., Makita, N., Roberts, L. J., II., Morrow, J. D., Takahashi, K., and Badr, K. F. (1993) *Am. J. Physiol.* **264,** C1619–C1624
21. Sevanian A., and Kim, E.(1985) *J. Free Radicals Biol. & Med.* **1,** 263–271